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Examining the importance of dynamics, barrier compression and hydrogen tunnelling in enzyme catalysed reactions.

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Abstract

Nuclear quantum mechanical tunnelling is important in enzyme-catalysed H-transfer reactions. This viewpoint has arisen after a number of experimental studies have described enzymatic reactions with kinetic isotope effects that are significantly larger than the semiclassical limit. Other experimental evidence for tunnelling, and the potential role of promoting vibrations that transiently compress the reaction barrier, is more indirect, being derived from the interpretation of *e.g.* mutational analyses of enzyme systems and temperature perturbation studies of reaction rates/kinetic isotope effects. Computational simulations have, in some cases, determined exalted kinetic isotope effects and tunnelling contributions, and identified putative promoting vibrations. In this review, we present the available evidence – both experimental and computational – for environmentally-coupled H-tunnelling in several enzyme systems, namely aromatic amine dehydrogenase and members of the Old Yellow Enzyme family. We then consider the relative importance of tunnelling contributions to these reactions. We find that the tunnelling contribution to these reactions confers a rate enhancement of ~1000-fold. Without tunnelling, a 1000-fold reduction in activity would seriously impair cellular metabolism. We therefore infer that tunnelling is crucial to host organism viability thereby emphasising the general importance of tunnelling in biology.

Keywords: aromatic amine dehydrogenase; kinetic isotope effect; Old Yellow Enzyme; promoting vibration.

Nomenclature

$\Delta\Delta H^\ddagger$	difference in activation enthalpy between H and D transfer ($\Delta H^{\ddagger D} - \Delta H^{\ddagger H}$)
ΔG^\ddagger	free energy of activation
κ	tunnelling contribution to the observed rate constant (Eq 2)
Γ	dynamic recrossing events (Eq 1)
AADH	aromatic amine dehydrogenase
CT	charge transfer
EIE	equilibrium isotope effect

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FMN	flavin mononucleotide
KIE	kinetic isotope effect
OYE	Old Yellow Enzyme
QM/MM	combined quantum mechanical/molecular mechanics
QMT	quantum mechanical tunnelling
r	distance
RHR	reductive half-reaction
RTD	representative tunnelling distance
RTE	representative tunnelling energy
MR	morphinone reductase
NAD(P)H	β -nicotinamide adenine dinucleotide (phosphate)
PETNR	pentaerythritol tetranitrate reductase
TTQ	tryptophan tryptophylquinone
V	(barrier) potential energy
ZPE	zero point energy

1. Experimental evidence for enzymatic hydrogen tunnelling

The hallmark of a hydrogen (either a proton, hydrogen atom, or hydride) transfer reaction that involves a degree of quantum mechanical tunneling (QMT) is the observation of an isotope effect that can not be explained by semiclassical transition state theory – *i.e.* that can not arise exclusively from the difference in zero point energy of *e.g.* typical C-H and C-D stretches (≈ 3000 and ≈ 2200 cm^{-1} , respectively). For example, an intrinsic primary H/D kinetic isotope effect (KIE; *i.e.* $k_{\text{H}}/k_{\text{D}}$) that is significantly greater than ≈ 7 at room temperature can not arise solely from ZPE differences. Secondary KIEs (where a neighbouring H is *e.g.* deuterated) that are larger than the equilibrium isotope effect (EIE) – the isotope effect on the equilibrium constant for the H-transfer step – can be similarly used as evidence for QMT [1]. Finally, exalted Swain-Schaad exponents [2] – which relate H/T to D/T isotope effects – can also be interpreted as arising from a reaction with a QMT component [3].

While electron tunnelling was first observed in proteins in the 1960's [4], the first evidence of QMT of hydrogen in an enzymatic reaction was described much later by Cha *et al* [5] who observed an inflated Swain-Schaad exponent on the yeast alcohol dehydrogenase catalysed oxidation of benzyl alcohol to benzaldehyde at room temperature. Dynamical aspects of H-tunnelling in enzymes was then inferred by Basran *et al* [6] and Kohen *et al* [7] from KIE studies with methylamine dehydrogenase and thermophilic alcohol dehydrogenase, respectively. Since then, considerable examples of thermally activated QMT during enzymatic H-transfer reactions have been reported (see *e.g.* refs [8-22]), suggesting that QMT may play a role in most H-transfer reactions. A more pertinent question, and one that cannot be answered easily by experiment, is whether the QMT contribution alters significantly the nature of the reaction.

2. Quantification of the QMT contribution to H transfer

There are several methods used to quantify QMT contributions to H-transfer reactions and typically they use semiclassical transition state theory (Eq.1) as a reference point.

$$k_{\text{TST}} = \Gamma(k_{\text{B}}T/h) \exp(-\Delta G^{\ddagger}/k_{\text{B}}T) \quad (1)$$

We briefly describe these approaches to prevent confusion over our nomenclature and to highlight some issues in the experimental quantification of such reactions. The tunnelling contribution, κ , can be defined as the magnitude of the rate enhancement due to tunnelling as observed in the apparent rate constant, k_{obs} :

$$k_{\text{obs}} = \kappa k_{\text{TST}} \quad (2)$$

If the observed rate constant comprises only contributions from rate constants that describe transfer via the transition state and by tunnelling (*i.e.* $k_{\text{obs}} = k_{\text{TST}} + k_{\text{tun}}$). Then:

$$\kappa = 1 + k_{\text{tun}} / k_{\text{TST}} \quad (3)$$

Similarly, the QMT contribution is sometimes quantified by defining the fraction (or %) of tunnelling in terms of the rate constant due to tunnelling relative to the observed rate constant:

$$f_{\text{tun}} = k_{\text{tun}} / k_{\text{obs}} = (\kappa - 1) / \kappa \quad (4)$$

Unless kinetic measurements can be performed at temperatures approaching 0 K (where the transition state reaction becomes frozen out), it is not possible to directly measure by experiment the individual contributions of k_{TST} and k_{tun} to k_{obs} . As almost all enzyme-catalysed reactions must be performed in a liquid aqueous solvent to allow free mixing of the enzyme and substrate (exceptions are the light-driven reactions in the membrane-bound photosynthetic reaction centres and soluble enzymes such as protochlorophyllide oxidoreductase), measurements are restricted to temperatures near room temperature. Consequently, κ values must generally be determined from tunnelling calculations.

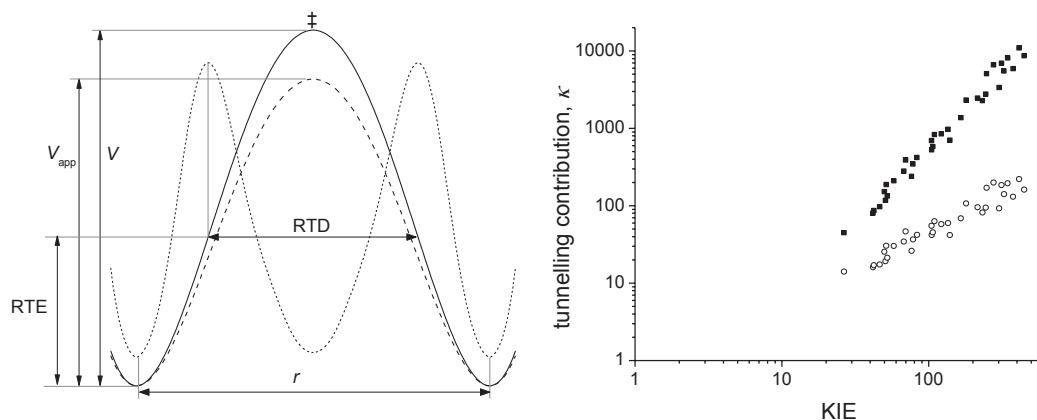


Fig.1. Modelling thermally-activated tunnelling. (Left) A theoretical reaction barrier (solid line) described using a quartic double well potential of height $V = 80 \text{ kJ mol}^{-1}$ (after ZPE correction for H) and width $r = 1.3 \text{ Å}$ and the Boltzmann-weighted tunnelling probability (PQ; dotted line) calculated using a WKB action as described in ref [23]. If the RTE is defined as the point along the reaction coordinate where PQ is maximal, then, for H-transfer, the RTE = 34 kJ mol^{-1} and the RTD = 0.77 Å . Conversely, the apparent barrier height, V_{app} , can be calculated from $k_{\text{obs}}^{\text{H}}$ to be 69 kJ mol^{-1} (dashed line). The apparent rate of H transfer, $k_{\text{obs}}^{\text{H}} = 5 \text{ s}^{-1}$ and the apparent enthalpy = 36 kJ mol^{-1} . $\kappa^{\text{H}} = 100$, $\text{KIE}_{\text{obs}} = 107$ and the apparent rate calculated from the RTE = $8 \times 10^6 \text{ s}^{-1}$. (Right) The relationship between κ^{H} (filled squares) and κ^{D} (open circles) and the KIE for the proton-transfer during the RHR of AADH with tryptamine (Fig 2). The κ and KIE values are taken from ref [23] and were calculated using a thermally-activated 1-dimensional WKB model with multiple reaction barriers obtained from QM/MM simulations. The apparent KIE and κ values can be estimated from a population-weighted mean of the plotted values [23].

If there is a significant QMT contribution to the reaction, the apparent (experimentally determined) reaction enthalpy (ΔH^{\ddagger}) or activation energy (E_a) will report on the apparent reaction barrier height, which may be considerably lower than the transition state (Fig.1). Consequently, at present it is not possible to directly benchmark enzymatic H-tunnelling calculations in terms of either the barrier potential energy or tunnelling correction. The observed activation enthalpy may be similar to the RTE (as defined in Fig.1), but it is not trivial to relate this term to either k_{obs} or κ . As a result, tunnelling calculations tend to be benchmarked against measured KIE values. In theory, there is a good correlation between calculated KIE and κ values (Fig.1). However, there is currently no ‘standard curve’ that will relate the magnitude of any measured KIE to a κ value. As an example, using comparable QM/MM calculations, we have determined similar κ values for the proton transfer step during the RHR of AADH with

tryptamine ($\kappa = 295$) [24] and the hydride transfer step during the RHR of MR with NADH ($\kappa = 350 \pm 250$) [25] but the observed room temperature KIEs on these reactions are 55 and 7, respectively. As a consequence, we would caution against using the magnitude of the KIE as a diagnostic of QMT. While there is good evidence to suggest that the QMT contribution is significant if the KIE is significantly larger than the semiclassical limit, the opposite is not necessarily the case. As the observed KIE is sensitive to the H-transfer distance, reorganizational processes, the catalytic commitments *etc.*, it is possible that the change in magnitude of a KIE effected by a change in environment (*e.g.* by altering the solvent composition, pressure, or by mutagenesis, *etc.*) may not be attributed solely to a change in QMT contribution to the reaction. The role of dynamics may further muddy the water.

3. The H-transfer distance and promoting vibrations

There has been considerable interest to correlate KIEs with the tunnelling distance. The tunnelling distance approaches the barrier width as the reaction becomes more non-adiabatic (*i.e.* κ approaches infinity). As the tunnelling distance is expected to be on the order of a bond length, X-ray crystallographic and NMR structural methods are not able to determine these distances with sufficient precision to be useful. Consequently, the tunnelling distance is usually determined either from computational analyses that have been benchmarked against KIE values [18,20,23,25] or by inference from experimental observation of the KIE [11,26]. A popular method, which treats the H-transfer as a non-adiabatic ‘full-tunnelling’ reaction, uses simple vibronic (‘Marcus-like’) models to describe the KIE in terms of the nuclear wavefunction overlap [23,27–32]. A feature of these models is that, as the tunnelling distance decreases, the wavefunction overlap increases, the rate of H-transfer increases and the KIE decreases. Consequently, these models are typically used to qualitatively interpret trends in experimental KIE data with respect to changes in the H-tunnelling distance [11,26,32,33]. There are several problems with using this approach, not least that H-transfers are unlikely to be non-adiabatic at room temperature. Despite this, we have found that, if a vibronic model is accurately parameterized (to describe the wavefunction) and the KIE is quite large, then the calculated tunnelling distances can be in good agreement with values determined from a thermally-activated tunnelling model based on the more accurate WKB action [23]. It is possible that this approach may, with some refinement, lead to reaction-specific ‘standard curves’ for the tunnelling distance-dependence of the KIE.

The coupling of dynamics that transiently reduce the barrier width to the H-transfer reaction is also a key question in the field [12,34–36]. The potential role of such ‘compressive dynamics’ has been contentious. Of particular interest is whether enzymes have evolved to use QMT to the best advantage, by coupling, when necessary, specific vibrations within the enzyme active site to the reaction coordinate. There are both real and semantic disagreements about the definition of such motions. We define a promoting vibration as a fast (ps) motion along the (H-transfer) reaction coordinate, consistent with ideas that have evolved from computational analysis of enzyme systems [20,23,37–42]. The timescale of these vibrations is dictated by $k_B T$ as the vibrations have to be readily activated thermally at room temperature. Consequently, the upper limit to the frequency of such vibrations is in the order of 100’s of cm^{-1} . Kamerlin and Warshel have argued that these vibrations have to be non-equilibrated (*i.e.* non-Boltzmann) [43]. Their reasoning is probably that if these vibrations were equilibrated, they would be accounted for by transition state theory and simply contribute to the barrier shape. However, if one considers a nonadiabatic reaction where the barrier shape is not explicitly considered (the barrier is considered to be infinitely high and only the width is important) any vibration that reduces barrier width is expected to enhance the rate of H-transfer, and is thus by definition ‘promoting’. Warshel has also argued that promoting vibrations are not catalytic [43,44]. It is important to note that this statement does not necessarily discount the possibility of such vibrations. Promoting vibrations could be general features of the reaction present also in an enzyme-free reference state (see Section 4). However, we are not aware of any experimental evidence to support this. Regardless, our view is that there is strong evidence to support (albeit indirectly) the existence of promoting vibrations in enzyme-catalysed H-transfer reactions.

Experimentally, the identification of promoting vibrations is extremely challenging as the THz region where these vibrations are expected to be found is a difficult region to probe spectroscopically. As such, experimental evidence for promoting vibrations during an enzymatic H-transfer reaction is often inferred from observations of KIEs with strongly temperature-dependent KIEs [7,13,14]. These data are generally interpreted [45,46] such that if the KIE is significantly temperature-dependent (ΔE_a or $\Delta \Delta H^\ddagger$ greater than $\sim 6 \text{ kJ mol}^{-1}$ *i.e.* the upper limit set by the Bell-correction model [47]), then the reaction is assisted by a promoting vibration. It is important to note, however,

that promoting vibrations have not been observed directly by experiment. Additional experimental methods supported by computational approaches are required for further insight. With this in mind, we now discuss studies of the H-transfer reaction in two classes of enzymes where we have found evidence that supports the existence of promoting vibrations: the reductive half-reactions (RHRs) of aromatic amine dehydrogenase (AADH) and two members of the Old Yellow Enzyme family [morphinone reductase (MR) and pentaerythritol tetranitrate reductase (PETNR)]. We then discuss the potential importance of these vibrations, and the more general significance of the QMT contribution to these reactions.

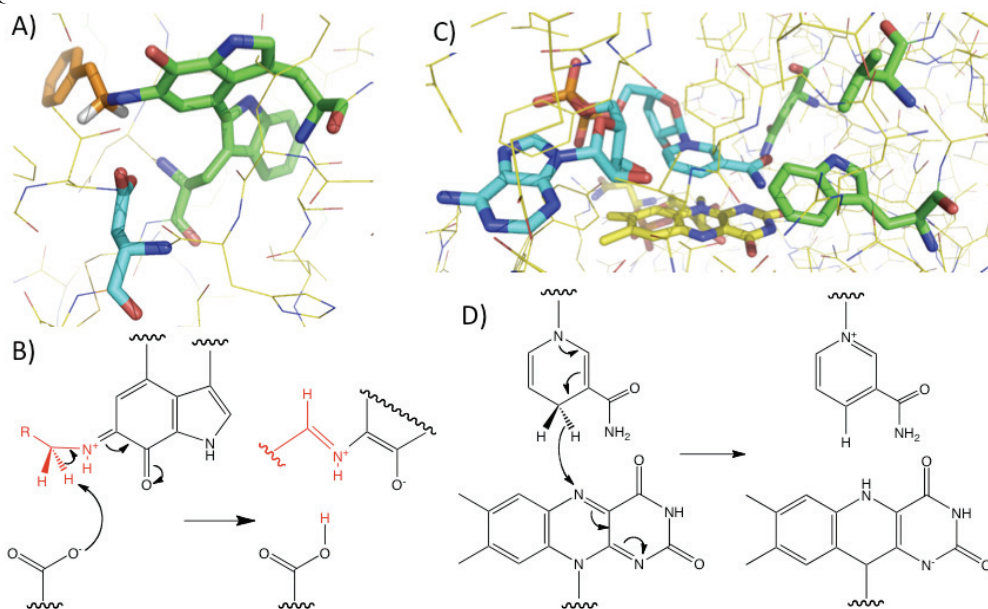


Fig.2. (A). Model of the AADH-phenylethylamine iminoquinone adduct based on the X-ray crystal structures described in ref [48]. The TTQ is shown in green, the substrate in orange, and the aspartate H-acceptor in light blue. (B). The proposed reaction mechanism of the proton transfer step during the RHR of AADH with an aromatic amine substrate. The portion of the TTQ adduct derived from the substrate is shown in red. (C). Model of the NADH-bound active site of MR based on the crystal structure with NADH₄. The FMN is shown in yellow, the NADH in light blue and key residues in the active site (Trp 105, Val 108 and Asn 186) in green. (D). The proposed mechanism of the hydride transfer step during the RHR of an OYE with NAD(P)H.

4. Aromatic amine dehydrogenase

AADH is a bacterial quinoenzyme that uses a TTQ (tryptophan tryptophylquinone) cofactor to oxidatively deaminate aromatic amine substrates (Fig.2). The enzyme is active toward a number of substrates but discussion here is restricted to two classes, tryptamine (2-(1H-indol-3-yl)ethanamine) and para-substituted phenylethylamines. The RHR is thought to be common to all substrates and is complex, involving ≈ 10 steps [18,49]. The rate limiting step with both tryptamine and phenylethylamine involves proton transfer from the substrate-derived portion of an iminoquinone adduct to an aspartate residue and has an observed KIE at room temperature of ≈ 55 with tryptamine [18] and 13-20 with phenylethylamines [48]. QM/MM variational transition state theory calculations on the proton transfer from tryptamine have shown that this reaction has a large QMT contribution to the reaction [18, 24]. While the tryptamine KIE is not measurably temperature-dependent, molecular dynamics simulations and spectral density calculations have identified a putative promoting vibration ($\sim 165 \text{ cm}^{-1}$) that transiently compresses the H-transfer distance [20]. Interestingly, the identified promoting vibration does not require large-scale dynamics of the protein scaffold, but is inherent to the proton donor (the iminoquinone adduct), and is thus probably not catalytic as the enzyme-free reference state would also contain this vibration. We have also shown that the apparent temperature-independence of the observed KIE on this reaction can be accommodated by vibronic models, as this promoting vibration will only impart a very weak temperature dependence on the KIE that may be experimentally

immeasurable over the accessible temperature range [20]. An interesting observation is that the apparent reorganisation energy for this reaction is very large ($> 200 \text{ kJ mol}^{-1}$).

The reaction of AADH with phenylethylamine substrates proceeds at a rate ≈ 10 -fold slower than with tryptamine [48]. We have not yet computationally characterised the QMT contribution to this reaction, but the magnitude of the KIE is sufficiently large to suggest that QMT is also significant. The temperature dependence on the KIE with various para-substituted phenylethylamines is variable with $\Delta\Delta H^\ddagger$ values ranging from 0 - 12 kJ mol^{-1} [48]. The origin of these, sometimes large, temperature-dependencies has yet to be established, but a promoting vibration similar to that observed during the reaction with tryptamine is expected. There is no obvious correlation between the magnitude of the KIE and $\Delta\Delta H^\ddagger$, suggesting that, if the QMT contribution is proportional to the magnitude of the 1° KIE, then the degree of environmental coupling (to the promoting vibration) is not well correlated to the QMT contribution. A qualitative driving force analysis revealed that the reaction rate constants and activation enthalpies are anti-correlated and the rate constant increases with increasing driving force [48]. This observation is compatible with a Marcus-type model for nonadiabatic H transfer (assuming the reaction is not occurring in the inverted region), but can also be accommodated by semiclassical transition state theory. Brinkley and Roth [50] have measured the driving force-dependence of the rate of hydride transfer during the RHR of glucose oxidase with glucose and also observed a strong driving force dependence on the rate of hydride transfer with a large apparent reorganisation energy of $\sim 280 \text{ kJ mol}^{-1}$. This reorganisation energy is similar to the value we estimated for the AADH reaction with tryptamine [20]. Large reorganisation energies may therefore be a common feature of H-transfer reactions. This might be expected, as reorganisation energies for electron transfer reactions are typically larger when significant bond rearrangement (as would be the case for any H-transfer reaction) is coupled to the reaction. It remains to be seen whether driving force experiments will provide a method for deconvoluting the contributions from the QMT and promoting vibrations in enzymatic H-transfers.

5. The Old Yellow Enzymes

The RHRs of the OYEs we discuss here involve the stereo-specific hydride transfer from the C4 *R*-hydrogen of β -nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) to the N5 atom of flavin mononucleotide (FMN; Fig 2). This reaction can be studied by pre-steady state (*i.e.* single turnover stopped-flow) methods and has been extensively characterized [13,26,51,52]. The H-transfer step is kinetically resolved from the preceding step(s) involving coenzyme binding and the reaction is essentially irreversible so the observed KIE approaches the intrinsic KIE [13, 26,51,52]. MR reacts with NADH, while PETNR can react with both NADPH and NADH. The temperature dependence of the primary (1°) KIE for these three reactions is shown in Fig 3A and associated kinetic parameters are given in Table 1. Based on these data we have inferred that the reactions of MR with NADH and PETNR with NADPH are assisted by promoting vibrations. The reaction of PETNR with NADH shows no evidence of environmental coupling, and we have suggested that the reduced rate of this reaction (relative to that with NADPH) arises due to the lack of a manifest promoting vibration [53]. We describe below additional experimental probes we have developed to characterise further these H-transfer reactions.

Hydrostatic pressure offers an alternative to temperature as an independent experimental parameter with which to study enzymatic reactions. Isaacs first used this approach to study the pressure-dependence of solution proton transfers [54] and Northrop was the first to use pressure to study enzymatic H-transfer reactions [55-62]. As the bond stretches that determine the ZPE [54,63,64] of the transferred H (*e.g.* the C-H stretches) have been shown to be invariant over the typical experimental range of several kbar [63] the observed pressure-dependence of the KIE has been interpreted as arising from the QMT contribution to the reaction. In principle then, the pressure-dependence of an isotope effect provides an excellent method to deconvolute the QMT contribution to the reaction. We have developed high-pressure stopped-flow methods to measure the variable pressure- and temperature-dependence of the 1° KIE on the RHR of MR in an attempt to deconvolute the contributions made by QMT and promoting vibrations to the H-transfer reaction [26]. We observed that, while the temperature-dependence of the KIE was not measurably pressure-dependent, the magnitude of both the rate constant and the 1° KIE (Fig 3B) increased with pressure. We have since measured the pressure-dependence of the PETNR reaction with both NADH and NAD(P)H (Fig 3B) [53]. A positive correlation is observed between the magnitudes of the temperature ($\Delta\Delta H^\ddagger$) and pressure (both on the activation volume $-\Delta\Delta V^\ddagger$ and on the compressibility $\Delta\Delta\beta^\ddagger$) dependence of the KIEs. It therefore appears that pressure, like temperature, is a good experimental probe of promoting vibrations.

A relatively large QMT contribution to the hydride transfer during the RHR of MR with NADH has been observed using QM/MM calculations [25]. As the PETNR/NADPH reaction is nearly identical to the MR reaction (both in terms of active site geometry and kinetics) it seems, by analogy, that the QMT contribution to this reaction is also likely to be large. The relative pressure-independence of the KIE on the PETNR/NADPH reaction then suggests that pressure may, in fact, be an unreliable probe of QMT. It would then seem that – much like the case of temperature dependencies of KIEs – while a strongly pressure-dependent KIE probably reports on a H-transfer with a significant QMT contribution, pressure-independent KIEs do not necessarily report on H-transfers that lack such a significant QMT contribution. As C-H stretches are largely pressure-independent, where does the pressure-dependence of a H-tunnelling reaction arise? The most obvious origin would be a change in barrier width with pressure – *i.e.* compression. Consequently, we feel that while pressure is an unreliable probe of QMT, it may be an excellent probe of compression and compressibility – phenomena that are directly linked to enzyme dynamics.

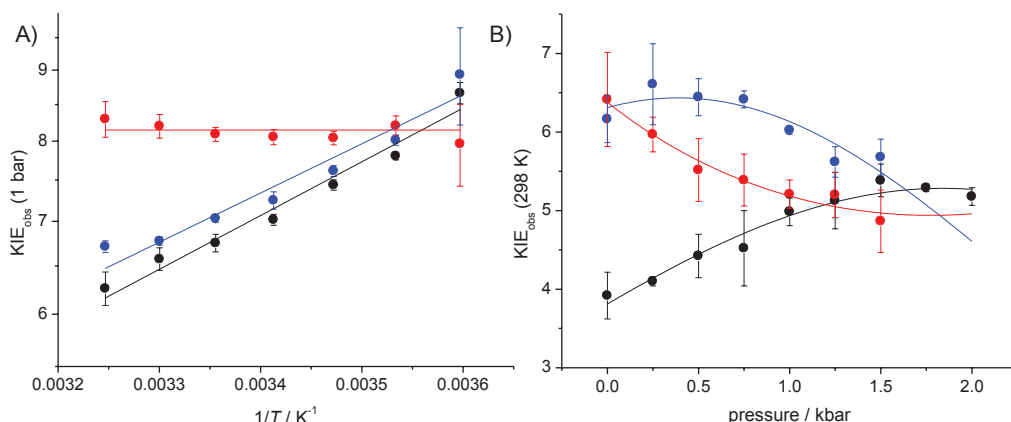


Fig.3. The temperature at 1 bar (A) and pressure at 298 K (B) dependence of the observed 1° KIE on the RHRs of MR with NADH (black), PETNR with NADH (red) and PETNR with NADPH (blue). The data are taken from refs [26, 53, 65] and the magnitude of the observed KIE on the MR reaction in (B) is reduced due to isotopic fraction [66] caused by the use of NAD²H with a ~6% isotopic (*i.e.* ⁴H) impurity [26].

Table 1. Selected thermodynamic and kinetic parameters for the hydride transfer during the RHR of some Old Yellow Enzymes (numbers in square brackets indicate primary source of data).

	enzyme, substrate		
	MR, NADH	PETNR, NADPH	PETNR, NADH
k_{obs} (298 K; s ⁻¹)	56.4 ± 0.08 [52]	33.5 ± 0.2 [53]	2.00 ± 0.02 [53]
1° KIE _{obs} (298 K)	6.8 ± 0.1 [65]	7.0 ± 0.1 [53]	8.1 ± 0.1 [53]
2° KIE _{obs} (298 K)	1.18 ± 0.02 [52]	1.17 ± 0.01 [52]	1.18 ± 0.01 [53]
$A^{\text{H}} : A^{\text{D}}$	0.4 ± 0.2 [65]	0.5 ± 0.1 [53]	5.2 ± 0.5 [53]
$\Delta\Delta H^{\ddagger}$ (kJ mol ⁻¹)	7.2 ± 1.5 [65]	6.5 ± 2.8 [53]	-1.1 ± 2.1 [53]
$\Delta\Delta V^{\ddagger}$ (cm ³ mol ⁻¹)	-8.8 ± 1.3 [67]	0.6 ± 6.1 [53]	5.4 ± 3.0 [53]
$\Delta\Delta\beta^{\ddagger}$ (cm ³ mol ⁻¹ kbar ⁻¹)	4.8 ± 1.1[67]	2.6 ± 6.5 [53]	-1.9 ± 3.2 [53]

In a recent review of our work, Kamerlin and Warshel [43] concluded that the pressure-dependence of the KIE on the reaction of MR with NADH arises due to an increase in the width of the reaction barrier with increasing pressure. This conclusion, which is counter to ours, would only be valid if the magnitude of the KIE reported directly on barrier width (as is the case for simple vibronic models). Unfortunately, these authors overlooked our experimental studies where we used absorption of the charge-transfer (CT) band formed between the FMN isoalloxazine and NADH nicotinamide rings within the active site MR as a ‘spectroscopic ruler’ [65,68]. The absorption of the CT bond will increase if the length of the bond decreases [69,70]. However, the absorption may

also change if the orientation of the two rings shifts (*e.g.* due to twisting of the two rings relative to each other about the plane orthogonal to the plane of the rings). Using fixed-pressure molecular dynamics simulations, we demonstrated that, in the MR active site, increasing pressure causes a reduction in the separation of the isoalloxazine and nicotinamide rings with a concomitant decrease of the donor-acceptor (C4-N5) distance by 0.17 Å over 2 kbar [68]. No significant twisting of the nicotinamide and isoalloxazine rings was observed. Consequently, we have concluded that, in the case of the MR reaction with NADH, pressure causes barrier compression. The caveat to this interpretation is that we have assumed that the equilibrium donor-acceptor separation in the reactant state reports on the barrier width. More recent calculations on both simple model systems, and on barriers calculated from QM/MM simulations of the AADH-tryptamine iminoquinone pre-tunnelling state [23] suggest that this assumption is reasonable. If we then model the pressure-dependence of the KIE on the MR reaction using a vibronic model (which is somewhat justified by the QM/MM calculations [25] described above) by assuming the pressure causes a decrease in the tunnelling distance, we find that the KIE decreases with increasing pressure. We were able to model the experimentally observed increase in KIE with decreasing barrier width only by incorporating into the modeling a pressure-dependent promoting vibration which becomes ‘stiffer’ (*i.e.* by increasing frequency) with pressure [26, 67]. This interpretation awaits a computational study to identify the atomistic nature of the promoting vibration and to characterise its pressure dependence (work that is currently in-hand).

α -secondary (2°) KIEs arise when a hydrogen neighbouring the transferred (1°) H (in the case of NAD(P)H, the pro-*S* H) is deuterated. Within the context of a semiclassical reaction, the magnitude of the 2° KIE is considered to indicate the progress of reactants to products at the transition state [71,72] with values falling between unity and the EIE. 2° KIE values that are larger than the EIE have been used as evidence for a QMT contribution to the reaction. [73-75]. While 2° KIEs have traditionally been measured using steady state methods [3,76] we have developed stopped-flow methods to measure these KIEs on the RHRs of OYE [52,53,65,70] and on the hydride transfer reaction in DHFR [77]. The 2° KIEs on the three OYE reactions discussed above, are identical (Table 1) and are significantly larger than the EIE, which has been measured as 1.13 for the conversion of NAD(P)H to NAD(P)⁺ [78]. Our interpretation of these data is that the 2° KIEs do not report on the promoting vibration, but rather on what we termed the ‘tunnelling-ready configuration’, which we define as the donor-acceptor geometry immediately preceding the H-transfer. In the context of a tunnelling reaction, this is the crossing point. We came to a similar conclusion while examining the temperature-dependence of the 2° KIE on the DHFR reaction [77].

In a recent study of selected active site mutants of MR, we were able to correlate the NADH/FMN CT absorption with the magnitude of the 2° KIE [65]. As the 2° KIE decreased, the magnitude of the CT absorbance increased and the temperature-dependence of the 1° KIE decreased. We interpreted these data by inferring that mutagenesis alters the equilibrium NADH C4-FMN N5 distance. The smaller 2° KIEs are associated with shorter heavy atom separation. We have also seen a similar trend in the pressure-dependence of the 2° KIE on the reaction of wild type MR with NADH. As the pressure increases (and the C4-FMN N5 distance decreases, see above), the magnitude of the 2° KIE was found to decrease [70]. In this case the magnitude of the 1° and 2° KIEs are anti-correlated, suggesting that, if the magnitude of the 1° KIE does reflect the QMT contribution (although see discussion above), then the magnitude of the 2° KIE is unlikely to do so.

6. Does the QMT contribution make a real difference in biology?

Although QMT during H-transfer is now widely appreciated in enzyme systems, the relative importance of the QMT contribution is still debated extensively, with a spectrum of current views held by key workers in the field [35,43,44]. Some (notably Warshel and co-workers) have downplayed the importance of nuclear QMT in enzyme catalysis [43,44]. In the main, this position is based on the strict chemical definition of catalysis (*i.e.* rate enhancement achieved by enzyme relative to a ‘reference state’) and that, in some cases, computed tunnelling contributions are found to be similar in the presence and absence of enzyme (though this is not a universally held view). While the catalytic effect can generate rate enhancements of $>10^{15}$, QMT contributions (*i.e.* κ values calculated from QM/MM simulations) determined both from our work [18,23,25] and that of others [79] are typically $\sim 2\text{--}10^3$. As even the larger contributions may be seen as a relatively small contribution to the overall catalytic effect, when compared to the reaction rate in absence of enzyme (*e.g.* 10^3 vs. 10^{15}), some authors have concluded that this lessens the importance of QMT [43,44]. However, the key issue, as we see it, is not the contribution to the overall catalytic effect, but the impact QMT has on biology. If the QMT contribution to these

reactions was theoretically ‘switched off’, then it follows that the reaction would proceed at a rate $\sim 10^3$ -fold slower than is observed experimentally (Eq.2). Consequently, as H-transfer reactions are ubiquitous, these QMT-less reactions would not support metabolic activity and cell viability would inevitably be compromised. For example, the substrates of both AADH and the OYE enzymes discussed herein are the primary carbon sources for their respective bacteria. These enzymes are unregulated in the presence of such substrates and, if the enzymes were 10^3 -fold less efficient (*i.e.* by a factor of κ , which for MR and AADH are $\approx 10^3$) [23–25, 80] then a comparable level of metabolic activity would only be maintained if 10^3 -fold more enzyme could be produced. This would place an unacceptable burden on these bacteria. The importance of QMT in biology then is not so much about ‘catalytic contribution’ in pure chemical terms (albeit an important chemical question involving multiple contributions including electrostatics, QMT, entropy *etc.*), but more about the life-sustaining role of QMT. We and others have shown that QMT is important for H-transfer in enzyme systems. By extension of the above arguments, it is likely that QMT is crucial to the life of an organism, therefore justifying fully the intensity of research activity in the field.

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